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The Effect of a Second Stress on Growth of Tetrahymena Pyriformis

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THE EFFECT OF A SECOND STRESS ON GROWTH OF TETRAHYMENA PYRIFORMIS

by

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This thesis submitted by Janice M. Granum in partial fulfillment of the requirements for the Degree of Master of Science in the University of North Dakota is hereby approved by the Committee under whom the work has been done.

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ABSTRACT

This research was undertaken to determine the effect on growth of Tetrahymena pyriformis of a single stress followed at some later time by a second stress, using temperature change and osmotic pressure change as stress factors.

Growth rate of T. pyriformis was determined under standard conditions of temperature and osmotic pressure (29°C and 100 milliosmols). A single stress consisting of a decrease in temperature to 20°C or an increase in osmotic pressure to 300 milliosmols was applied to an exponentially growing culture and its effect noted. After a recovery period a second, different stress was applied to the culture and its effect noted.

From results of these experiments the following conclusions were reached: (1) Either single stress caused a brief lag, followed by resumption of exponential growth at a lesser rate. (2) The order in which the stresses were applied did not change their overall effect on growth. (3) The most obvious effect of the double stress was a lengthening of the lag period. (4) The organisms adapted more quickly to the two stresses when a recovery period was allowed between stresses than when both were applied simultaneously.

INTRODUCTION

General Considerations

Tetrahymena pyriformis is a ciliated protozoan which has been found in most areas of the world where collections from fresh water habitats have been made. The organism was seen by most early microscopists who observed protozoa and was identified by many synonyms before the 1950's when the name *Tetrahymena pyriformis* became generally accepted. The generic name is derived from the fact that the mouth of the organism has four membranous structures--a right hand undulating membrane and three left hand membranelles. The species name comes from the typical pyriform, or pear-shape, of the cell (7).

Tetrahymena is motile by means of 17-22 rows of cilia. The organism is generally about 30 by 50 microns, but there is much variation in size among different strains as well as within the same strain when growing under different conditions (5). Generally, the morphology and the nutritional requirements are similar among the many strains, although there are variations in such things as temperature optimum and growth rate. These appear to depend upon the natural habitat from which the organism was isolated (7).

In nature *Tetrahymena* feeds on bacteria. In the laboratory it can be grown axenically in ordinary or

chemically defined media. The necessary constituents of a chemically defined medium include 11 amino acids, 7 B vitamins, guanine, uracil, and several inorganic ions. Neither fatty acids nor carbohydrates are required for growth but both may be stimulatory (8).

Nutritional studies by G. W. Kidder (8) and his associates in the 1940's furnished the major contribution in the establishment of the amino acid and vitamin requirements of Tetrahymena. They also investigated the inorganic ion requirements of the ciliate. Slater (20, 21) contributed to the establishment of cobalt and magnesium requirements.

Because it can be cultivated axenically with ease, it is adaptable to many growth conditions, it has a relatively short generation time, and it is similar metabolically to mammalian cells, Tetrahymena is frequently chosen for investigation in the laboratory, not only in nutritional and metabolic studies but in many other areas. A review by Corliss (6) indicates that in recent years the majority of papers dealing with Tetrahymena are in the fields of biochemistry and physiology, particularly with regard to enzyme systems and metabolic pathways. The literature concerning Tetrahymena includes many articles in genetics, cytogenetics, cytology, and systematics.

Effect of Temperature on Growth of Tetrahymena pyriformis

The influence of temperature on biological systems or processes is of universal interest. Many investigators

have examined the effects of temperature on T. pyriformis (13, 14, 24).

One basic type of experiment frequently seen is simply a determination of how temperature effects the division rate of microorganisms.

Phelps conducted a series of investigations on growth of Tetrahymena in pure culture (11, 12, 13). The third paper in this series was concerned with the effect of temperature on division rate. He determined growth curves during the exponential growth phase at a series of temperatures between 7.82° and 28.58°C. He found that when his data were plotted either by the Van't Hoff-Arrhenius equation or by Q_{10} there were breaks giving three distinct groups from 7.82°-12.30°, from 12.30°-20.03°, and from 20.03°-28.58°. The Q_{10} value was 9.7 at the low temperatures, 3 in the middle group, and 1.5 at the high temperatures. This indicates a greater change in generation time per degree change in temperature in the lower range than at higher temperatures. The fission time in hours for the strain Phelps used ranged from 2.88 at 28.58° to 26.6 at 7.82°.

Prescott (14) investigated the relation between multiplication rate and temperature in two strains of T. pyriformis, namely, HS and GL. Strain HS had previously been isolated from a hot spring and had a considerably higher optimum temperature than strain GL. Actually, HS had a faster multiplication rate at any of the temperatures Prescott used. Prescott also checked the adjustment of T. pyriformis HS to

a change in temperature. He found that the effect on an exponentially growing culture of a sudden shift from 18.4° to 27.7°C was first a slight growth synchrony, after which the rate quickly leveled off at that characteristic for the organism at 27.7°. The apparent lag or readjustment phase was about one hour. This was calculated by extrapolating the straight slope of the points at 27.7° back to the abscissa. The line did not pass through the point where the temperature shift occurred but fell one hour to the right of this point. Prescott points out that the adjustment of T. pyriformis to a new temperature apparently occurs very rapidly, and does not require several days as suggested by Phelps.

Especially interesting is the classic experiment of Scherbaum and Zeuthen (17) in which they induced synchronous division in a population of T. pyriformis by intermittent heat shocks over a period of 6½ hours. They found that the repeated exposure of the cultures to one-half hour at 34°C followed by one-half hour at 29°C brought about synchronous division of 85 per cent of the cells. This synchrony was soon lost, however, and after two or three waves of diminishing synchronous division the culture returned to normal random multiplication.

Since then many papers have been published on the subject of synchronous division and the effects of various shocks on cell growth and division. A review on the subject by Scherbaum (18) presents his hypothesis on likely sites of

metabolic control with regard to cell division. Evidence indicates that the generation time of a cell is more readily affected by the environment than is cell size. For example, T. pyriformis subjected to intermittent heat shocks can be almost completely prevented from dividing, but the total mass of the culture will increase to 65 per cent that of a normal control. In keeping with this observation is data from Thormar (23) showing that cell sizes of Tetrahymena are smallest at temperatures between 22° and 27°C. Below 20° and above 30° they are larger, indicating that growth is less sensitive to temperature than is reproduction. Since cells can be blocked from division by either heat or cold, Scherbaum concluded that either heat or cold affect a denaturation of an enzyme system.

The main reason for interest in division synchrony was the hope that an entire population of cells in essentially the same phase of their life cycle would be available for biochemical study. It appears that synchronous division does not necessarily indicate synchronous growth of the individual cells, since once the culture is relieved from the synchronizing agent only a brief period of division synchrony results. The division times are quickly randomized because of the natural large variation in generation times.

Effect of Osmotic Pressure on Growth of T. pyriformis

Osmotic pressure is another physical phenomenon which effects the growth of microorganisms. Much of the investigation

on the effects of osmotic pressure has involved bacteria. Most bacteria can survive and grow over a fairly wide osmotic pressure range. Some actually require high osmotic pressure for growth (9).

Osmotic pressure may be expressed in osmols or milliosmols (mos) as well as in atmospheres. An osmol is equal to 22.4 atmospheres and is the osmotic pressure of a one molal solution of a non-electrolyte at $0^{o}C$.

Osmotic pressure is actually a pressure difference--just as freezing point depression is a temperature difference. Osmotic pressure may be defined as that pressure which must be applied to a solution to keep that solution in equilibrium with a pure solvent when the solvent is separated from the solution by a membrane which is permeable to the solvent (4).

Osmotic pressure is difficult to measure but may be calculated when freezing point depression is known, since both are colligative properties of solutions.

The natural environment of Tetrahymena is a fairly dilute one with an osmotic pressure much lower than that of the cell cytoplasm. The organism maintains water balance with the functioning of a contractile vacuole which ejects water from the cell (22). Indications are that many protozoa can adapt to changes in osmotic pressure. Closely related species of protozoa have been found in fresh and in salt water, and many fresh water species have been shown to be tolerant of osmotic pressures higher than that of their

normal habitat (10).

In 1939 Loefer (10) investigated the tolerance of certain fresh water ciliates, including Tetrahymena, to either sudden or gradual changes to media of higher salt concentration. He demonstrated the acclimatization of Glaucoma piriformis (Tetrahymena pyriformis) to a 70% Van't Hoff solution. A 100% Van't Hoff solution[1], an artificial sea water, was reported by Loefer as having a total salinity of 3.97%. Natural sea water has a total salt content of 3.2-3.3% (26). In a series of 15 transfers Loefer gradually increased the salt concentrations of the media to which the organisms were transferred. He found that by this method T. pyriformis developed a tolerance to a 70% Van't Hoff solution. There was a change in morphology in the higher salt concentrations and also a decrease in average cell size. When the organisms were returned to the original low salt medium (1% Van't Hoff medium) they returned to their normal size and appearance within several days. Loefer did not determine generation times nor overall population increase. His criteria for adaptation to the new media were simply motility and viability.

Browning (1) used sucrose to increase the osmotic pressure of culture medium for Tetrahymena. He found that by increasing the calcium ion concentration of the medium

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[1]This artificial sea water was composed of 0.625 M solutions of salts mixed in the following proportions: $CaCl_2$, 1 part; $MgCl_2$(NaCl?), 7.8 parts; $MgSO_4$, 3.8 parts; KCl, 2.2 parts.

he could diminish the inhibitory effect of increased osmotic pressure. He also found that increasing the sucrose concentration while holding calcium concentration constant caused increased generation times. The highest concentration of sucrose he used was 0.4 molar (approximately 400 mos). At this concentration the generation time was 20 hours as compared to 6½ hours with 0.1 M sucrose (100 mos). He noted that the culture in 0.4 M sucrose had decreased by one-third and was apparently not growing at the end of 61 hours incubation, but after 165 hours it had increased by several generations and continued to multiply. This suggests that if survival of a species in a particular medium is to be determined, a large number of organisms should be observed over an extended time interval.

Evidently T. pyriformis can adjust fairly rapidly to temperature changes and is capable of survival and growth in media having a wide osmotic pressure range. Reynolds and Wragg (16) reported growth in media with osmotic pressures of from 2 to 9 atmospheres (equivalent to approximately 90 to 400 mos). While there is considerable information on the effects of varying either of these environmental factors, there is apparently no information available on the effect of combined stresses consisting of changes in both temperature and osmotic pressure.

In nature organisms such as Tetrahymena encounter combinations of physical stresses. This research was undertaken to determine the effect on growth rate of

T. pyriformis strain S of a single stress followed at some later time by a second stress, using temperature change and osmotic pressure change as stress factors.

METHODS AND MATERIALS

Organism

The organism used in this study was *Tetrahymena pyriformis* strain S, which had been obtained from Dr. G. R. Seaman and maintained for several years in the departmental culture collection.

The medium used for maintenance of the culture during the experimentation period was the same as the basic medium used for growth curves. Fifty-ml screw top tubes, each containing 20 ml of the sterile medium, were inoculated with 0.1 ml of a three-day-old culture. The tubes were incubated for 18 hours at room temperature and then refrigated at 4°C. Since the cells multiply slowly at 4°, these cultures could be kept without transfer for about two months.

When an experiment was to be started a fresh tube of sterile medium was inoculated from one of the refrigated cultures, incubated at room temperature for 48 hours, and used as the immediate source of organisms for the experimental flasks.

Media

All media, dilution blanks, pipettes, and other glassware used were sterilized by autoclaving for 15 minutes at 20 pounds pressure.

The medium used for culture maintenance and for experimental growth curves consisted of 1% peptone (Difco), 1% glucose, and 0.1% yeast extract (Difco) in distilled water (w/v). In experiments where an osmotic stress was applied, mannitol was added to the basic medium to increase the osmotic pressure. The pH of this medium was about 6.5.

Counts were made by a modification of the procedure for viable plate counts of protozoa (24). Sterile 9 ml and 99 ml water blanks were used for dilutions except for counts from cultures growing at increased osmotic pressure. When dilution of those cultures was necessary, dilution blanks were made of the same medium in which the culture was growing. Plating medium consisted of distilled water containing 1% skim milk (Difco) and 0.5% agar. Various concentrations of skim milk and agar were tried, but the above concentrations yielded plates that were easiest to count and most reproduceable. Double strength skim milk and agar were sterilized separately, cooled to 45°C in a water bath, and mixed together in equal amounts just before plates were poured.

Several plating techniques were tried and abandoned before the media and method described for obtaining plate counts was adopted.

Standard Conditions

Before experiments involving stresses were performed it was necessary to establish the growth rate of T. pyriformis under standard conditions of temperature and osmotic pressure.

Since 29°C had been established previously as the optimum for growth of strain GL (14), this was chosen as the standard temperature. The osmolarity of the basic medium was 100 ± 5 milliosmols (mos) as measured on the Fiske Osmometer. This was chosen to be the standard osmolarity. The pH of the medium was 6.5 and did not change significantly during the course of an experiment. Prescott (15) has shown that within the range from 5.65 to 8.40 pH has little effect on growth rate of Tetrahymena (the change in generation time was only one hour in going from pH 5.65 to 7.34, the optimum for the strain he used). Slight changes in pH during an experiment were therefore not considered significant.

In order to insure an adequate oxygen supply 500 ml screw-top Erlenmeyer flasks containing 100 ml of medium, or 1000 ml flasks containing 200 ml of medium, were used for all growth curves. The small amount of medium in proportion to the flask size gave a large surface to volume ratio and allowed free diffusion of oxygen into the medium. The depth of the medium was approximately 12-13 mm. The caps of the flasks were not tightly sealed.

Growth Curve Procedure

Basic medium was made, dispensed into screw-top Erlenmeyer flasks, sterilized, and cooled to room temperature. A 10^{-1} dilution of 48 hour culture was used as the inoculum and was added at the rate of 1 ml per 100 ml of medium. This dilution was found necessary to keep the inoculum small and to get approximately the same number of organisms into

each flask of an experimental series.

After inoculation the flasks were placed in a 29°C incubator and samples were removed aseptically with a sterile pipette for plate counts. Time intervals for plating varied and are given in the section on results. Before a sample was removed the flasks were swirled to insure even distribution of the protozoans.

As the population increased dilutions of the samples were necessary to obtain countable plates. In all cases these dilutions were made so that 1 ml aliquots could be plated. For example, rather than use 0.1 ml of a 10^{-2} dilution for the 10^{-3} plate, a 10^{-3} dilution was made and 1 ml of it plated. This should contribute to accuracy, since there is less chance of error in pipetting 1 ml than in pipetting 0.1 ml.

The cooled skim milk and agar were mixed and poured into the Petri plates, which were swirled gently to distribute the organisms uniformly. The plates were incubated at 29° for 15-18 hours. Usually, plates having at least 20 colonies and preferably those with 30-250 colonies were counted with the aid of a Quebec Colony Counter. In the semi-opaque medium each viable protozoan produces a clear zone of hydrolysis. See Plate 1. All plates were made in triplicate.

Experiment to Determine an Inert Sugar for T. pyriformis Strain S

Sucrose and mannitol were chosen as possible agents

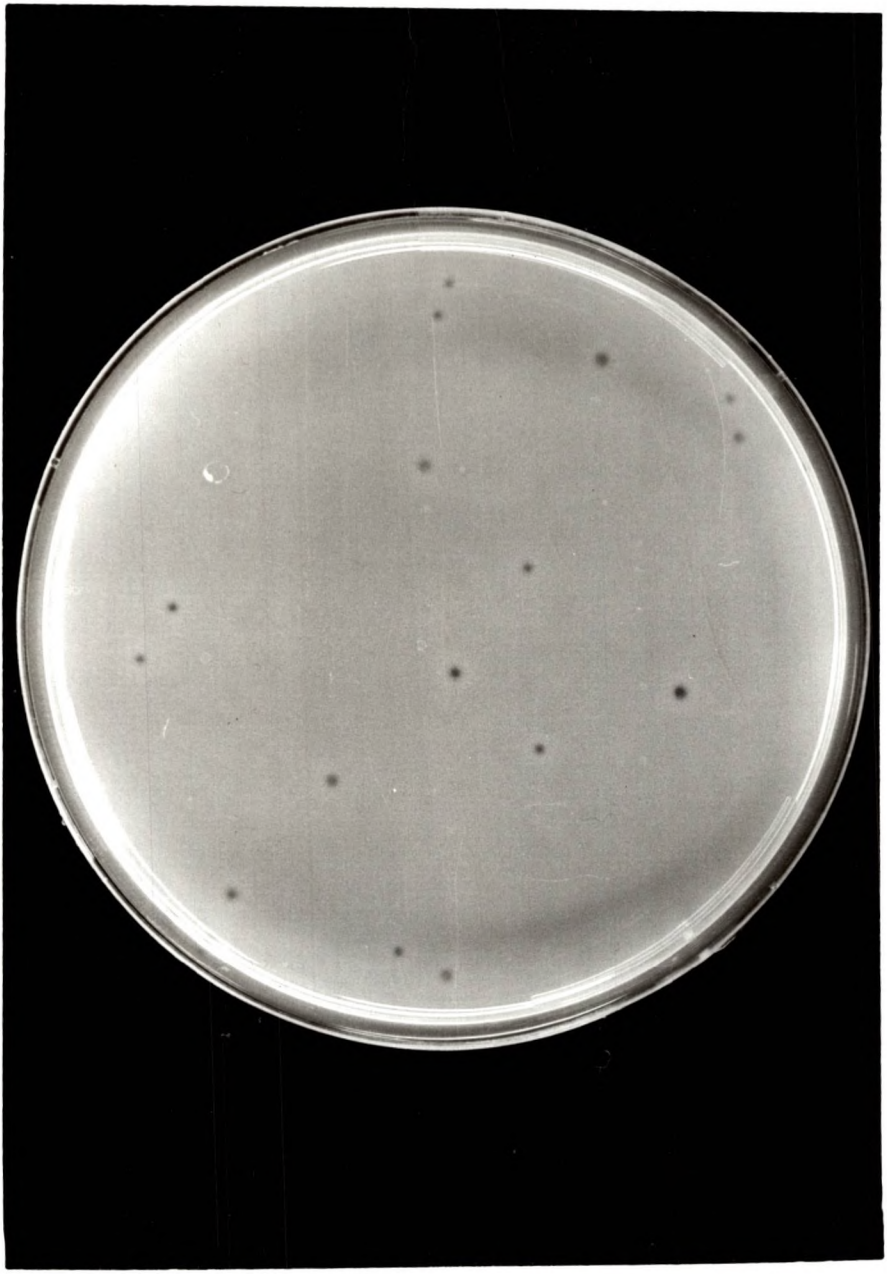

Plate 1.-Colonies of *T. pyriformis* on skim milk agar.

for increasing the osmotic pressure of the medium. They are non-electrolytes, are readily soluble in water, are non-toxic and apparently are generally not utilized by ciliates (19).

Utilization of a carbohydrate involves release or uptake of gases. For measurement of gas exchange a manometric experiment is suitable, therefore this type of experiment was set up to determine whether or not mannitol or sucrose would be utilized by T. pyriformis.

To get a sufficient number of cells, cultures were grown in a biphasic medium (24). Two sets of 3 flasks were prepared. In the first set each liter flask contained 300 ml of 1% peptone, 1% glucose, 0.1% yeast extract, and 2% agar overlaid with 100 ml of the same medium without the agar. The second set was like the first with the exception that glucose was omitted. The 6 flasks were each inoculated with 1 ml of the refrigated T. pyriformis culture and incubated at 29°C for 72 hours. The liquid was decanted from the flasks into centrifuge tubes and centrifuged at 2500 rpm for 10 minutes. The broth was quickly poured off the cells. The contents of the 3 tubes grown with glucose were pooled by resuspending in 25 ml of phosphate buffered distilled water. The contents of the 3 tubes grown without glucose were likewise pooled.

Manometric flasks were set up in duplicate for each of the sugars to be tested and for distilled water. One set of flasks was prepared using cells grown with glucose and one set using cells grown without glucose. For each

flask 2½ ml of cell suspension was placed in the main compartment and 0.5 ml of distilled water, 5% glucose, 5% mannitol, or 5% sucrose was placed in the sidearm. One flask from each pair also had fluted filter paper moistened with 5 drops of 20% potassium hydroxide in the center well. The atmosphere was air.

The flasks were attached to manometer tubes, placed in position in the 30° water bath, and allowed to equilibrate. The contents of the sidearms were tipped in and readings were taken at one hour intervals for 9 hours. The amount of oxygen consumed and carbon dioxide liberated was calculated for each flask and the R. Q. values calculated.

Experiments to Determine Osmotic and Temperature Stresses

Growth curves were determined for temperatures of 31.5° and for 20°C. An attempt was made to grow the organisms at 32 and 33°, but it was found that they ceased to multiply after a generation or two at these temperatures. The multiplication rate appeared to be about the same at either 20 or 31.5°. This rate was somewhat less than that of the optimum temperature. Since an incubator at 20° was readily available, this temperature was chosen as the stress temperature.

A preliminary experiment was set up in which sets of 8 tubes each containing 6 ml of basic medium plus varying amounts of mannitol or sucrose were inoculated with T. pyriformis, incubated, and observed for growth. In the following discussion of method only mannitol will be

included, but an identical procedure was followed using sucrose. The amount of mannitol necessary to raise the osmolarity of the medium a specific amount was calculated and the correct amount added to the respective tubes. For example, since the osmolarity of the stock medium was about 100 milliosmols (mos), a 0.15 molal addition of mannitol would bring the total osmolarity to approximately 250 mos. Molal additions ranging from 0.075 to 1.2 were added to the series of 8 tubes. An inoculated control and an uninoculated blank were included. A like series of uninoculated tubes were prepared to use in measurement of freezing point depression so that an accurate initial osmolarity could be determined for each concentration.

After inoculation the cultures were incubated at 29° and observed at 16, 24, 48, 72, and 96 hours. An attempt was made to read the tubes in a Klett colorimeter to quantitate the growth somewhat, but the turbidity of tubes displaying growth was not great enough to show significant differences in optical density. The results were therefore recorded simply as "growth" or "no growth". Freezing point depression was measured on 2 ml aliquots from each inoculated tube and from the corresponding uninoculated tube.

Growth curves were then determined at increased osmotic pressures. Five-hundred ml Erlenmeyer flasks were used, each containing 100 ml of basic medium and varied amounts of mannitol. The flasks were inoculated and freezing point depression was measured on a 2 ml sample from each

flask. One ml samples from each flask were also plated immediately after inoculation and at approximately 4 to 6 hour intervals for the duration of the experiment. Freezing point depression was measured on a sample from each flask when an experiment was terminated until it was established that no significant change in osmolarity of the media occurred during the period involved.

One additional preliminary experiment was carried out to determine the effect of a sudden increase in osmotic pressure on a culture growing exponentially under standard conditions. In this experiment it was necessary to add mannitol in such a way that a high concentration would not develop in any part of the medium. This was accomplished by using double strength mannitol in the basic medium and slowly, with constant gentle swirling, adding a volume of double strength mannitol equal to that of the growing culture. Since the exact volume of the culture was not known after several mls had been removed for plate counts, the procedure was to mix the culture carefully, measure 50 ml of it into a sterile graduated cylinder, and transfer it to a clean, sterile Erlenmeyer flask. Fifty ml of the mannitol medium was added to this in the manner described. This, of course, decreased the population by one-half.

All sampling or adding of mannitol to the medium was carefully done to avoid contamination. Cultures were observed for bacteria frequently and any contaminated flasks discarded.

Second Stress Experiments

Second stress experiments were continued for 48 hours. This time interval permitted sampling the cultures several times during the exponential phase to establish the slope of a standard growth curve before the first stress was applied. It also allowed enough time for the culture to adjust to the first stress before the second was applied.

By starting with few (approximately 25-30) protozoans per ml the entire experiment could be conducted at population levels that in a normal growth curve would have been in the exponential phase. A control flask was kept at standard conditions and plated over the same time interval.

Since these experiments were of longer duration than previous ones 1000 ml flasks and 200 ml of medium were used so that the volume would not be decreased significantly by the greater number of samples removed. For the increase in osmotic pressure 100 ml of culture was transferred to a sterile 1000 ml flask and 100 ml of double strength mannitol in basic medium was added in the manner previously described.

Calculation of Slopes

Slopes of all growth curves were calculated by determining the equation for a straight line through the sampling points by the method of least squares. The first point on each curve that was apparently due to a lag was not included in the calculation of the slope.

RESULTS

A control flask consisting of a culture of T. pyriformis growing under standard conditions was included with each experiment performed. The logarithm of the number of ciliates versus time was plotted for each of these controls, and the equation for the best straight line through the exponential phase points calculated by the method of least squares. These equations are listed in Table 1. For the actual plate counts from which the equations were derived see the Appendix.

As can be seen from the variation in the y intercept, the number of organisms was not the same at the beginning of all experiments. To determine whether or not the slope was independent of the inoculum size in this group of controls, the y intercept versus the slope was plotted. The scatter diagram indicated no apparent correlation between slope and y intercept. This is in agreement with the observations of Phelps (11), who found that the size of the inoculum did not effect the lag time nor the exponential growth rate of Tetrahymena when the inoculum came from an exponential phase culture.

The mean slope for the controls is 0.080 and the standard deviation 0.0085. The generation time for each of

TABLE 1.-Exponential growth rate of *T. pyriformis* under standard conditions

Experiment number	Equation	Generation time (hours)
I	$y = 0.092x + 1.3$	3.2
II	$y = 0.073x + 0.4$	4.0
III	$y = 0.085x + 1.4$	3.5
IV	$y = 0.080x + 1.1$	3.8
V	$y = 0.083x + 1.2$	3.6
VI	$y = 0.074x + 1.6$	3.9
VII	$y = 0.087x + 1.2$	3.5
VIII	$y = 0.066x + 1.4$	4.4

the standard growth curves is also included in Table 1. The mean generation time is 3.7 hours and the standard deviation is 0.37 hours.

Table 2 shows the results of a manometric experiment designed to determine what carbohydrate would be suitable to increase osmotic pressure without adding a utilizable substrate. Obviously cells cultured with glucose yielded no information about utilization of sugars. These cells should have been washed with buffer to remove the residual glucose-containing medium. Repeated washing and centrifugation was not done because of a reported undesirable effect on respiration of protozoan cells (3). Results from cultures grown without glucose indicate that glucose was utilized (as was previously known) and that mannitol and sucrose apparently were not.

Several experiments were conducted to determine the concentration of mannitol or sucrose which would constitute an osmotic stress for Tetrahymena. It was desirable to increase the osmotic pressure enough so that there would be an obvious effect on growth rate, but not enough to cause complete cessation of division. The method for these experiments was previously described. The results of the preliminary turbidimetric experiment are shown in Table 3. A 0.55 M addition of either sucrose or mannitol to the basic medium stopped growth during the 96 hour period of observation. A 0.3 M addition of either allowed growth but the 0.3 M and 0.275 M sucrose were more inhibitory than mannitol

TABLE 2.-Manometric determination of an inert carbohydrate for T. pyriformis

	Oxygen consumed	Carbon dioxide liberated	R.Q.
Culture grown with glucose			
Distilled water control	1308	1189	0.91
Glucose	1340	1193	0.89
Mannitol	1341	1219	0.91
Sucrose	1259	1101	0.88
Culture grown without glucose			
Distilled water control	888	694	0.78
Glucose	962	900	0.94
Mannitol	871	664	0.76
Sucrose	874	686	0.78

TABLE 3.-Turbidemetric experiment to show the effect of various concentrations of mannitol or sucrose on growth of T. pyriformis

Addition to basic medium	Observation		Osmotic pressure in milliosmols	
	2 days	4 days	Initial	Final
0.55 M mannitol	-	-	*	*
0.30 M mannitol	-	-	410	414
0.275 M mannitol	+	+	340	324
0.15 M mannitol	+	+	253	252
0.075 M mannitol	+	+	173	174
0.55 M sucrose	-	-	*	*
0.30 M sucrose	-	±	429	412
0.275 M sucrose	-	±	341	340
0.15 M sucrose	+	+	260	258
0.075 M sucrose	+	+		177
None (control)	+	+	96	95
None (blank)	-	-	96	95

*This solution did not freeze readily, therefore freezing point depression was not measured.
+ = Growth.
- = No growth.

in these concentrations. This increased inhibition must have been due to other than an osmotic effect. Mannitol was therefore chosen as the carbohydrate to increase the osmotic pressure.

Growth in test tubes is somewhat inhibited because of the unfavorable surface to volume ratio. The next step was to grow cultures of T. pyriformis in Erlenmeyer flasks with basic medium containing various concentrations of mannitol. Several such experiments were conducted to arrive at an osmolar level that would be inhibitory without being lethal. Results of these experiments are given in Tables 4 through 8.

The results in Tables 4 and 5 indicate that in medium with an osmotic pressure of 160 or 200 mos growth was actually stimulated. The medium with an osmotic pressure of 339 mos appears to be somewhat inhibitory, while 378 and 418 mos are highly inhibitory.

Tables 6, 7, and 8 show that medium with an osmotic pressure of 300 mos or above is inhibitory. At the higher osmotic pressures the size of the inoculum apparently has an effect on the length of the lag phase. Tables 6, 7, and 8 include duplicate series, one of which was incubated at 29°C and the other at 20°C. It appears that either 300 or 335 mos constitute an osmotic stress, one which is not extremely severe but does slow reproduction. The organisms will multiply in medium of either osmolarity while incubated at 29° or at 20°C.

TABLE 4.-Growth curves of T. pyriformis at 29°C in media of various osmotic pressures

Hour	protozoans per ml			
	C(99 mos)*	160 mos	200 mos	340 mos
0	65	84	66	34
3	84	100	100	43
6	110	160	130	63
9	140	190	170	71
20	340	550	670	200
24	390	750	930	250
29	550	990	1200	470
32	730	1400	1900	460
Final osmolarity	99	158	200	338

*Basic medium

TABLE 5.-Growth curves of T. pyriformis at 29°C in medium of various osmotic pressures

	protozoans per ml				
Hour	C(112 mos)*	160 mos	205 mos	378 mos	418 mos
0	27	22	23	23	20
4	41	34	35	20	8
8	86	70	77	19	8
12	140	150	140	38	20
22	320	550	390	110	30
26	480	720	700	120	60
30	670	1000	950	90	60
34	670	1200	1200	140	110

*Basic medium

TABLE 6.-Growth curves of *T. pyriformis* at 29° and 20°C in media with various concentrations of mannitol

	protozoans per ml							
	29°				20°			
Hour	C(102 mos)*	305 mos	340 mos	382 mos	C(102 mos)*	305 mos	335 mos	383 mos
0	3	2	2	2	4	6	4	3
4	4	5	3	2	4	4	3	4
8	9	5	4	3	8	5	5	3
12	29	8	5	3	14	4	2	1
19	83	22	20	6	42	9	4	2
23	110	25	24	16	55	5	1	0
27	220	52	43	22	100	2	1	0
31	450	79	58	21	240	5	6	1

*Basic medium

TABLE 7.-Growth curves of T. pyriformis at 29° and 20°C in basic medium with various concentrations of mannitol

	protozoans per ml					
	29°			20°		
Hour	C(95 mos)*	300 mos	335 mos	C(95 mos)*	300 mos	335 mos
0	24	23	19	22	22	19
4	34	28	23	25	19	18
8	130	49	45	36	22	22
12	310	110	69	83	30	26
18	990	320	130	220	33	20
24	2300	400	200	680	56	10
30	7200	590	290	1700	76	290
36	23000	850	450	4100	140	620
42	49000	1500	610	10000	1900	1400
48	130000	1300	910	25000	3400	2200
Final pH	6.2	6.0	6.0	6.0	6.1	6.0

*Basic medium

TABLE 8.-Growth curves of T. pyriformis at 29° and 20°C in media with an osmotic pressure of 300 mos

	protozoans per ml			
	29°		20°	
Hour	C(103 mos)*	300 mos	C(103 mos)*	300 mos
0	18	17	18	16
4	25	19	19	18
8	55	34	27	13
11	160	52	36	27
19	710	100	140	66
23	1300	150	240	4
29	3200	200	660	56
35	9800	360	2000	45
42	20000	570	6800	110
48	45000	510	9900	290

*Basic medium

The second stress experiments were designed to show the effect on an exponentially growing culture of a single stress followed, after a recovery interval, by a second stress. Three such experiments were carried out. Each consisted of a series of three flasks: a control, an experimental flask in which the temperature stress was applied first and the osmotic stress second, and an experimental flask in which the osmotic stress was first and the temperature stress second. Data for the growth curves from these experiments are given in Tables 9 and 10.

Equations were calculated for the best straight line through the group of points for each segment of the growth curves using time (in hours) versus the logarithm of the number of organisms. These equations, as well as the average slope for each segment, are shown in Table 11. The first point from experiments 1 and 2 was omitted when calculating the slope since there was an apparent lag at the beginning of these two experiments.

Lines having slopes equal to the average for each segment of the growth curves were plotted using the average y intercept to position the lines on the coordinate system. See Figures 1 and 2.

The equations for Figure 1 (temperature stress first) are: for the first 15 hours, $y = 0.083x + 1.3$; for 15 to 27 hours, $y = 0.038x + 2.0$; and for 27 to 48 hours, $y = 0.020x + 2.1$. The broken line from C to D shows the immediate decrease in organisms, due partly to the dilution of the

TABLE 9.-Effect of a double stress on growth rate of *T. pyriformis* (temperature stress followed by osmotic stress)

Experiment 1		Experiment 2		Experiment 3	
Hour	organisms per ml	Hour	organisms per ml	Hour	organisms per ml
0	44	0	30	0	26
3	53	3	26	4	44
6	98	6	49	8	76
9	160	9	84	10	110
		12	150		
15*	600	15*	360	16*	340
17	840	17	470	18	430
19	840	19	490	20	410
21	1100	24	780	24	640
24	1300				
27**	1600	27**	1200	28**	1100
27	760	27	570	28	500
28	760	28	600	29	450
30	470	29	600	30	470
32	1200	31	440	32	310
37	1100	35	880	35	560
41	1100	39	910	41	780
45	1700	43	450	45	930
48	2500	48	1400		

*The temperature was changed from 29° to 20°C after this plating.
**The osmolarity of the basic medium was raised to 300 mos after this plating.

TABLE 10.-Effect of a double stress on growth of *T. pyriformis* (osmotic stress followed by temperature stress)

Experiment 1		Experiment 2		Experiment 3	
Hour	organisms per ml	Hour	organisms per ml	Hour	organisms per ml
0	60	0	24	0	27
3	69	3	26	4	58
6	120	6	38	8	100
9	220	9	90	10	140
15	780	12	160		
16*	890	15*	400	16*	420
16	320	15	190	16	170
17	290	16	180	17	220
19	390	17	180	18	180
21	550	19	250	20	270
24	650	24	580	24	360
28**	970	27**	640	28**	580
30	710	29	740	30	480
32	840	31	870	32	710
37	1000	35	850	35	520
41	1300	39	960	41	880
45	1600	43	1200	45	980
48	2500	48	2200	49	700

*The osmotic pressure was increased from 100 mos to 300 mos after this plating.
**The temperature was changed from 29° to 20°C after this plating.

TABLE 11.-Equations for segments of growth curves from second stress experiments

Temperature stress first			
Growth phase:	Initial	After temperature stress	After osmotic stress
Experiment 1	y = 0.087x + 1.5	y = 0.029x + 2.4	y = 0.027x + 2.0
Experiment 2	y = 0.092x + 1.1	y = 0.042x + 1.9	y = 0.013x + 2.4
Experiment 3	y = 0.070x + 1.4	y = 0.043x + 1.8	y = 0.020x + 2.0
Average slope:	0.083	0.038	0.020

Osmotic stress first			
Growth phase:	Initial	After osmotic stress	After temperature stress
Experiment 1	y = 0.087x + 1.6	y = 0.044x + 1.8	y = 0.027x + 2.0
Experiment 2	y = 0.099x + 1.0	y = 0.053x + 1.4	y = 0.022x + 2.2
Experiment 3	y = 0.073x + 1.4	y = 0.043x + 1.6	y = 0.019x + 2.2
Average slope:	0.087	0.047	0.023

culture and partly to the initial shock. C to E' shows the probable population if dilution had not been necessary.

Similarly, in Figure 2 (osmotic stress first) the equations plotted are: for the first 15 hours, $y = 0.087x + 1.3$; for 15 to 27 hours, $y = 0.047x + 1.6$; and for 27 to 48 hours, $y = 0.023x + 2.1$. In Figure 2 the broken line from B to C represents the decrease in protozoans due to dilution and initial osmotic shock. The broken lines from C to D' and D' to E' indicate where the population would probably have been without dilution.

Consideration of the average slopes given in Table 11 and illustrated in Figures 1 and 2 leads to two conclusions: first, as a single stress the temperature change decreased the growth rate slightly more than did the osmotic pressure change; and second, the overall effect of the double stress was the same regardless of whether the temperature stress or the osmotic stress was applied first.

Another question to be considered was whether or not these two stresses would have a greater effect on growth if they were applied concurrently. Table 12 gives results of three growth curves of T. pyriformis cultured under such conditions. The cultures were started at 20° and 300 mos with a population of 38-40 organisms per ml and were sampled over a 48 hour period. None of the cultures increased in numbers by more than two generations during the first 30 to 35 hours. Apparently the longer lag was due to the change in temperature and osmotic pressure since a culture started

FIGURE 1.-Effect of a double stress on growth rate of T. pyriformis (temperature stress followed by an osmotic stress). Designation of line segments: AB, initial exponential growth; BC, growth after the first stress; DE, actual growth after the second stress; CE', apparent growth after the second stress.

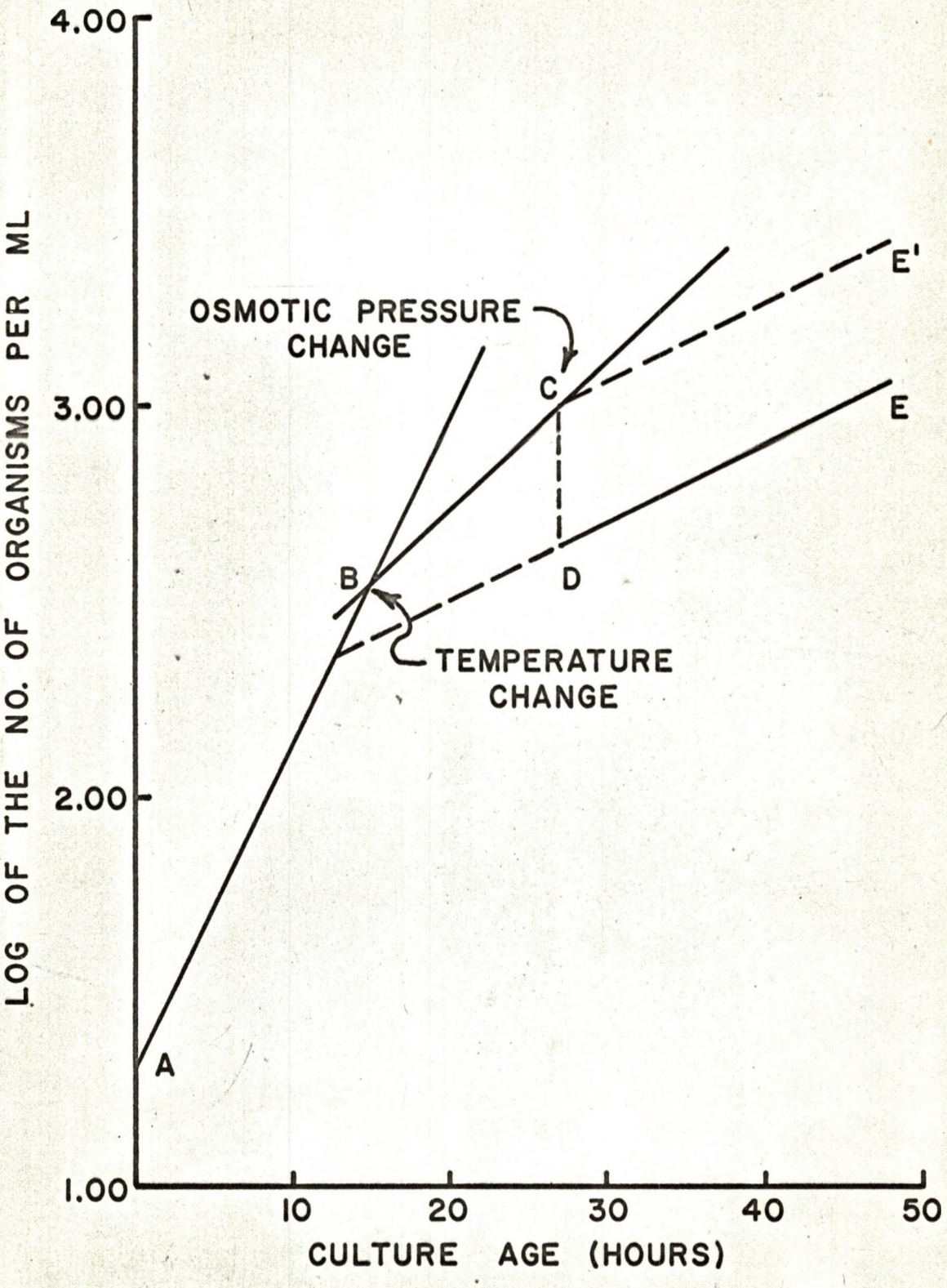
4.00
3.00
2.00
1.00
LOG OF THE NO. OF ORGANISMS PER ML
OSMOTIC PRESSURE CHANGE
TEMPERATURE CHANGE
A
B
C
D
E
E'
10
20
30
40
50
CULTURE AGE (HOURS)

FIGURE 2.-Effect of a double stress on growth rate of T. pyriformis (osmotic stress followed by a temperature stress). Designation of line segments: AB, initial exponential growth; CD, actual growth after the first stress; BD', apparent growth after the first stress; DE, actual growth after the second stress; D'E', apparent growth after the second stress.

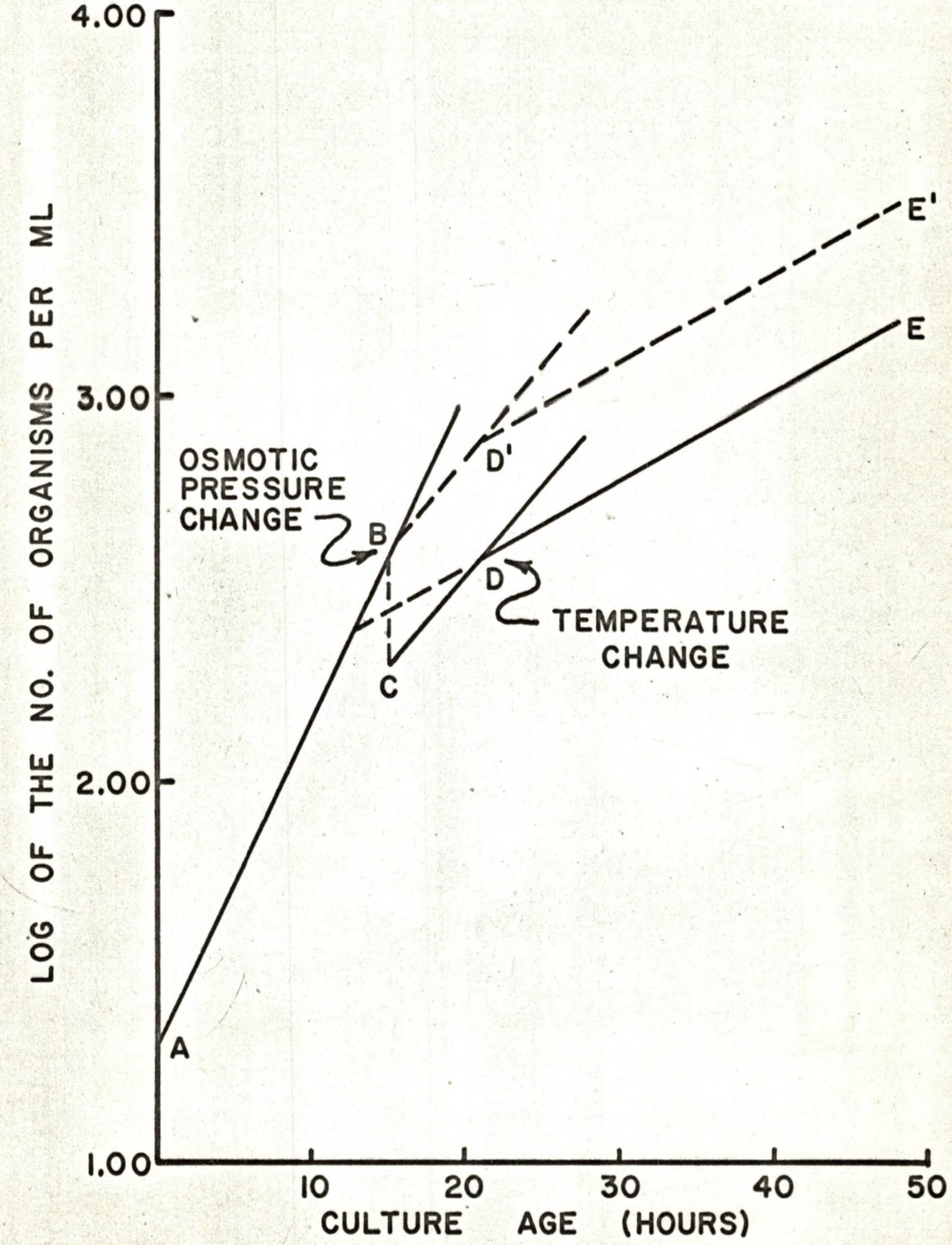
4.00
3.00
2.00
1.00
LOG OF THE NO. OF ORGANISMS PER ML
OSMOTIC PRESSURE CHANGE
TEMPERATURE CHANGE
A
B
C
D
D'
E
E'
10
20
30
40
50
CULTURE AGE (HOURS)

TABLE 12.-Growth of *T. pyriformis* at 20°C and 300 mos

Hour	protozoans per ml	Hour	protozoans per ml	Hour	protozoans per ml
0	38	0	38	0	40
4	28	4	32	3	33
8	35	8	33	6	30
12	38	12	40	9	29
21	73	21	36	15	29
25	21	25	40	21	35
28	89			24	26
33	90			28	69
43	680			32	56
48	900			37	65
				41	420
				45	910
				48	1500
Slope: (first 21 hours)	0.0158		0.00084		0.00011

with a similar inoculum under standard growth conditions shows a lag time of 4 hours or less. After 30 to 35 hours had elapsed the rate of multiplication increased. For a time the rate actually appeared greater than that normally found with standard growth conditions. Unfortunately, the cultures were not sampled over a long enough time to establish an exponential growth rate at 20° and 300 mos. In comparing these growth curves to those of the second stress experiments only the first 15 to 20 hours need be considered since in those experiments the sampling time elapsing after the second stress was applied was never longer than 15 to 20 hours. Slopes were calculated for the first 18-21 hours because all cultures were not sampled at exactly 20 hours. The average slope for the three experimental curves was 0.0055. This is considerably less than the average for the final segment of the second stress experiments (0.022), suggesting that the double stress had a greater inhibitory effect when both stresses were applied at once.

DISCUSSION

The ciliated protozoan T. pyriformis strain S was used to study the effect on growth of a double stress consisting of a temperature stress and an osmotic stress. In this study growth was considered as an increase in population numbers, only one of several ways of quantitating growth. Changes in individual cell sizes and in population mass were not investigated. Viable plate counts were used as the method for measuring population numbers.

A previous investigator has demonstrated that the effect on exponentially growing Tetrahymena of a change in temperature is a brief lag followed by resumption of logarithmic growth (with a transient synchronous division) at the rate characteristic of the new temperature (14). Other investigators have shown that an increase in osmotic pressure by addition of fairly large amounts of carbohydrate to the growth medium increased the generation time of T. pyriformis (1, 16).

For microorganisms there exists an optimal temperature and osmotic pressure for growth. While the optimal temperature for several strains of T. pyriformis has been established, there is no such information available for osmotic pressure. With bacteria the optimal osmotic pressure range

is usually fairly wide (9).

From results of this investigation it appears that the optimal osmotic pressure for T. pyriformis strain S is near 160 to 200 mos. In media having these osmolarities growth was more rapid than in the standard (100 mos) medium used. A medium with an osmotic pressure of 300 mos, however, increased the generation time over that found when the organisms were grown in the standard medium.

A decrease in temperature from 29° to 20°C or an increase in osmotic pressure from 100 to 300 mos caused an increase in generation time and could therefore be considered stresses.

There were three phases in each of the double stress growth curves. Each will be discussed separately.

The first phase in each case was the period during which a normal logarithmic growth rate for the individual culture was established. The slopes for this section of the growth curves all lie within two standard deviations of the mean slope for normal growth established under standard conditions with the exception of Experiment 2 (osmotic stress first), where the exponential growth rate was somewhat faster. In this experiment both cultures appeared to have a somewhat shorter generation time than expected. The relative effects of the stresses appeared to be the same as in the other two experiments, however.

The second phase of each experiment was that following application of a single stress, consisting of either a

temperature or an osmotic pressure change. The effect of a decrease in temperature of 9°C was first a short lag (about two hours) followed by resumption of logarithmic growth at a somewhat lesser rate. During the first two hours after changing to the 20° incubator the culture continued to grow at the 29° rate. This was the time necessary for temperature adjustment of the medium. The third and fourth hours showed a lag and after this time logarithmic growth resumed at the lower rate.

The immediate effect of a single stress consisting of an osmotic pressure change from 100 to 300 mos was also a lag of about two hours before the cells commenced regular division at what appeared to be a logarithmic rate. The effect of either single stress, then, was a brief lag followed by exponential growth at a new rate. Prescott (14) has observed that almost any sudden shift in environment may bring on a lag phase in an exponentially growing culture of Tetrahymena, and that this lag is roughly proportional to the magnitude of the shift. Data from the second phase of any of the growth curves appears to be in agreement with Prescott's observation.

The third phase of the curves, that following application of a second stress is more difficult to evaluate. There was some difficulty in counting cultures which had been subjected to the double stress. Three plates were made for each point, and the average of these was taken to establish each point on the growth curves. In all cases except those

from cultures sampled after the double stress these counts agreed well. In double-stressed cultures, however, there appeared to be discrepancies in that plates of the same dilution occasionally yielded widely different numbers of cells. In some cases the numbers of cells actually decreased over the first several platings. Browning et al (2) found that fragility of T. pyriformis increased in medium of higher salt concentration so that shaking of the cultures caused some cell breakage. The osmotic pressure of the medium they used was not given. It is doubtful that an osmotic pressure of 300 mos was causing the rupture of the cells, since the 300 mos cultures which were incubated at 29° did not show this effect. Another interesting phenomenon occurred when a culture which had been growing at 300 mos and 20° was changed to the 29° incubator. Within 1½ hours the population had increased by almost two generations, showing that the cells were ready to divide but for some reason did not do so when the culture was at 20°.

Since the counting method did have these limitations it was decided that the effects could perhaps best be represented by averaging the experimental data from the three experiments into two graphs. These graphs were discussed previously in the section on results, and are pictured in Figures 1 and 2. As was previously stated, the single stress of temperature appeared to have a greater effect when applied to a growing culture than the osmotic stress. The overall effect of the two stresses appears to be the same

regardless of which came first.

Another factor to be considered is whether or not these stresses have the same effect on growth when both are applied concurrently as when one is applied several hours before the other. Comparison of data from cultures growing at 20°C and 300 mos with that segment of the second stress experiments after both stresses had been applied leads to the conclusion that these stresses are better tolerated when applied separately.

In evaluating the effects of a second stress of either temperature or osmotic pressure, the first and probably the most significant effect on growth appears to be on the length of the lag phase. In all cultures the number of cells was static or decreased for about 15 hours after the second stress had been applied. An even longer lag period was found when a culture was started at 20° and 300 mos. In these cultures 25 to 35 hours passed before there was any significant increase in numbers. Toward the end of the 48 hour period all but one of the cultures had begun to multiply. Unfortunately, these cultures were not observed for a longer time so that the exponential growth rate for a culture of T. pyriformis at 20° and 300 mos could have been established. The experimental data does show that the second stress has a far greater effect as far as lag is concerned than does the first stress.

SUMMARY

This research was undertaken to determine the effect on growth of T. pyriformis of a single stress followed at some later time by a second stress. Temperature change and osmotic pressure change were used as stress factors. Growth was considered as increase in population numbers and was measured by viable plate counts.

The growth rate of T. pyriformis was established in a medium consisting of 1% glucose, 1% peptone, and 0.1% yeast extract in distilled water (w/v), with incubation at 29°C. The osmotic pressure of this medium was 100 ± 5 milliosmols (mos). These conditions were designated as "standard."

Growth curves were determined at 31.5° and 20°C, and 20° was selected as a stress temperature.

Growth curves were determined with various concentrations of mannitol added to the standard medium to increase the osmotic pressure. An increase of osmotic pressure to 300 mos was chosen as an osmotic stress.

The effect of a second stress on growth of T. pyriformis was determined using either a temperature stress followed by an osmotic stress or an osmotic stress followed by a temperature stress.

From the results of these growth experiments the

following conclusions were reached.

1. Either single stress caused a brief lag, followed by resumption of exponential growth at a lesser rate.

2. The order in which the stresses were applied did not change their overall effect on growth.

3. The most obvious effect of the double stress was a lengthening of the lag period.

4. The organisms adapted more quickly to the two stresses when a recovery period was allowed between stresses than when both were applied simultaneously.

APPENDIX

EXPERIMENT I

Hour	No. of organisms per ml	Log. no. of organisms per ml
5	50	1.699
11	220	2.342
20	1500	3.176
24	2700	3.431
29	8800	3.944

EXPERIMENT II

Hour	No. of organisms per ml	Log. no. of organisms per ml
4	4	0.602
8	9	0.954
12	29	1.462
19	83	1.919
23	110	2.041
27	220	2.342
31	450	2.653

EXPERIMENT III

Hour	No. of organisms per ml	Log. no. of organisms per ml
4	34	1.532
8	130	2.114
12	310	2.491
18	990	2.996
24	2300	3.362
30	7200	3.857

EXPERIMENT IV

Hour	No. of organisms per ml	Log no. of organisms per ml
0	12	1.079
4	23	1.362
10	130	2.114
18	440	2.644
24	1300	3.114
28	2200	3.342
34	6000	3.778

EXPERIMENT V

Hour	No. of organisms per ml	Log. no. of organisms per ml
4	25	1.398
8	55	1.740
11	160	2.204
19	710	2.851
23	1300	3.114
29	3200	3.505
35	9800	3.991

EXPERIMENT VI

Hour	No. of organisms per ml	Log. no. of organisms per ml
4	50	1.699
8	170	2.230
12	500	2.699
21	1600	3.204
24	3000	3.477
28	4400	3.644
32	6900	3.839

EXPERIMENT VII

Hour	No. of organisms per ml	Log. no. of organisms per ml
3	19	1.279
6	45	1.653
9	88	1.945
12	160	2.204
15	460	2.663
19	1100	3.041
24	2800	3.447
28	4700	3.672
31	6700	3.826
35	10000	4.000

EXPERIMENT VIII

Hour	No. of organisms per ml	Log. no. of organisms per ml
0	22	1.342
4	44	1.644
8	83	1.919

(EXPERIMENT VIII CONTINUED)

Hour	No. of organisms per ml	Log. no. of organisms per ml
10	99	1.996
16	330	2.519
20	560	2.748
24	1200	3.079
28	2100	3.322
32	3500	3.544
35	5200	3.716

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